Biochemical and Biophysical Research Communications **256,** 147–155 (1999)
Article ID bbrc.1999.0274, available online at http://www.idealibrary.com on IDEAL®

Lipophilins: Human Peptides Homologous to Rat Prostatein

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Received January 25, 1999

Lipophilin components A, B and C are human homologues of prostatein, the major secreted protein of rat prostate. This report describes their cDNA sequences, tissue expression and chromosomal localization. Lipophilin gene products were widely expressed in normal tissues, especially in endocrine-responsive organs. The gene for lipophilin C (also called mammaglobin b) is located on chromosome 11q12-q13.1, near the mammaglobin gene, a homologue overexpressed in many breast cancers. The lipophilin B gene resides on chromosome 10q23, a region deleted in many tumors, and the lipophilin A gene is on chromosome 15q12-q13.

Prostatein—also called "estramustine binding protein," and "prostatic steroid-binding protein"—is the major secretory glycoprotein of the rat ventral prostate gland [1], where its synthesis is controlled dramatically by androgens [2]. Rat prostatein is composed of three different peptides, called C1, C2 and C3. These form covalent C1:C3 and C2:C3 heterodimers whose noncovalent association forms tetrameric (C1:C3|C3:C2) prostatein molecules [3]. Prostatein C1 and C2 genes have similar exon/intron arrangements and high sequence homology, consistent with an origin via gene reduplication [4].

Human counterparts of the C1, C2 and C3 components of prostatein were described only recently , when a novel heterodimeric protein in human tears was identified and sequenced [5]. One of its components, lipophilin A, was homologous to the C1 and C2 components of prostatein. The other, lipophilin C, was homologous to rat prostatein C3 and to mammaglobin, a protein overexpressed in some human mammary carcinomas [6]. Lipophilin C was also identified independently, and named mammaglobin B [7]. We now report the cDNA cloning of the previously isolated lipophilins A and C and of lipophilin B, a newly discovered homologue of lipophilin A. We also describe the chromosomal localization of these genes, and describe their expression in normal tissues.

MATERIALS AND METHODS

The primers used in the following experiments are listed in Table 1. Based on the amino acid sequence of lipophilin A [5], we designed degenerate primers, P1 (sense, corresponding to KMEVKKCV) and P2 (antisense, complementary to KIAEKCD) and used them to amplify a human lachrymal gland cDNA library. We used a TOPO TA kit (Invitrogen, Carlsbad, CA) to subclone the ≈95 bp PCR product, whose deduced sequence was identical to lipophilin A. To get 5′ side, we used a specific antisense primer, P3, and the vector SK primer to amplify the lachrymal gland library. Amplified 320 bp product was subcloned and sequenced, yielding 5′-side untranslated region, signal sequence, and most of the mature Lipophilin A cDNA sequence. To complete the sequence, we amplified the library with 5′-side sense primer P4, and vector T7 primer, and sequenced the cloned PCR fragments.

We used the 51 bp signal sequence of lipophilin A to search the EST data base and found an incomplete sequence (THC 210918) with high homology. Using this sequence, we designed two primers: P8 (sense) and P9 (antisense). 3′ RACE PCR were carried out to amplify human uterus cDNA (Marathon-Ready, Clontech, Palo Alto, CA). P9 and adapter primers were used to get 5′-side cDNA, and P8 and adapter primers were used to get 3′-side cDNA. There was a 150-bp sequence overlap between the two PCR products.

Lachrymal cDNA library DNA was amplified with vector SK primer and degenerate antisense primer P11, complementary to lipophilin C amino acids EKTINSD. The PCR product (≈145 bp) included some lipophilin C 5′ untranslated sequence, signal sequence and part of the mature peptide. To obtain the full sequence, 5′ side sense primer P12 and vector T7 primer were used to amplify lachrymal cDNA. When the PCR fragments were subcloned and sequenced, they provided the full 496-bp cDNA sequence shown below.

Total human RNAs prepared from multiple human organs were purchased from Clontech (Palo Alto, CA). cDNA (20 μl) was synthesized from 1 μg of each total RNA with an Advantage RT-for- PCR kit (Clontech, Palo Alto). The PCR primers and the sequence locations shown in Table 1 were based on sequences reported herein or elsewhere [6, 8–10]. The human β-actin control amplimer set (Clontech)

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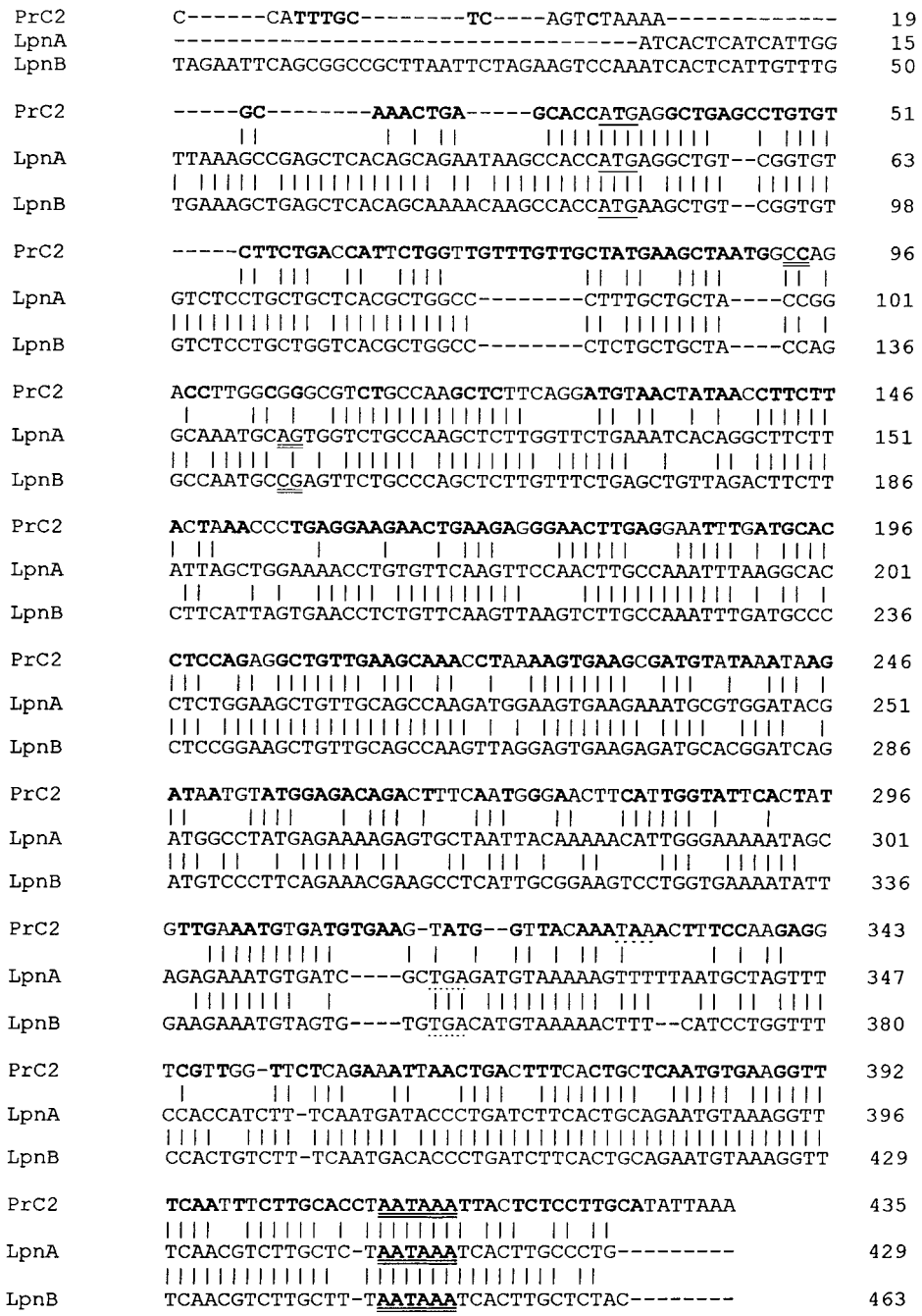

FIG. 1. cDNA sequences of lipophilins (Lpn) A and B and rat prostatein (Pr) C2. Identical bases are connected by a vertical line. Bases that are identical in rat prostatein components C2 and C1 (not otherwise shown) are bolded in the PrC2 sequence. The ATG start codons are underlined and the bases surrounding the signal sequence cleavage site are doubly underlined. TAA stop codons are delineated by dotted underlining and the polyadenylation signals of lipophilins A and B are bolded and double-underlined.

generated a 838 bp product. PCR was performed with an automated DNA thermal cycler for 35 cycles. Annealing temperatures from 55°C to 70°C were chosen according to the different primer sets. We used a master regent mix in each experiment to ensure tube to tube consistency in PCR reactions. Reaction products (15 μl) were visualized after electrophoresis in 1.4% agarose gel containing 0.5 μg ml^{-1} ethidium bromide. Because several of the cDNA sequences we studied were highly homologous, we designed a different primer set and repeated the PCR analyses for each cDNA, confirming the findings presented (data not shown).

We analyzed the data quantitatively by electrophoresing PCR products in agarose, transferring them onto nylon membranes (DuPont, Boston, MA), and hybridizing them with digoxigenin labeled specific probes (P7 for lipophilin A, P10 for lipophilin B, P14 for lipophilin C, P17 for human mammaglobin, and P20 for human uteroglobin). Labeling, washing and detecting were using the non-

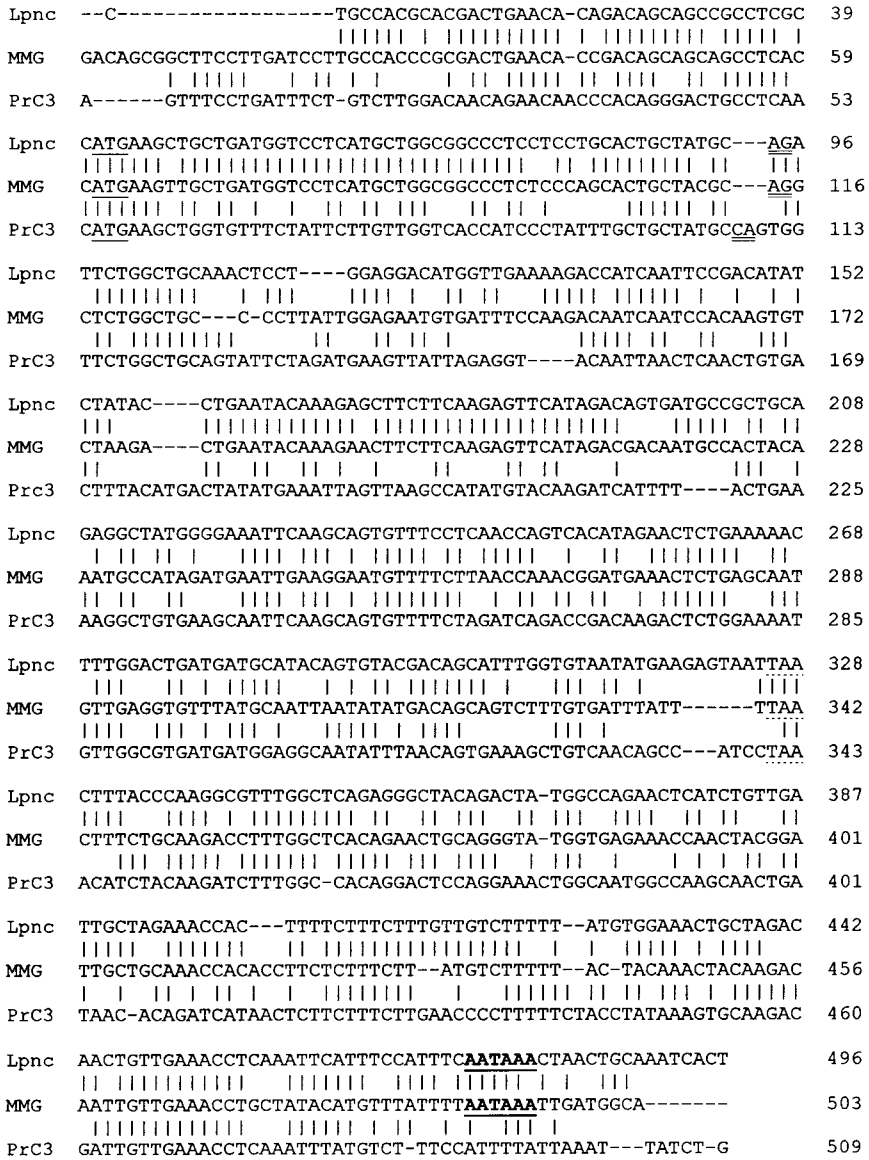

FIG. 2. cDNA sequences of lipophilin C (LpnC), human mammaglobin (MMG), and rat prostatein C3 (PrC3). Identical bases are connected by a vertical line. ATG start codons are underlined, and bases that bound the signal sequence cleavage site are double-underlined. Stop codons are indicated by dotted underlining and the polyadenylation signals of lipophilin C and mammaglobin are bolded and underlined.

radioactive DIG High Prime DNA Labeling and Detection Starter kit (Boehringer Mannheim, Indianapolis, IN), per the manufacturer's protocol. Light emission after alkaline phosphatase-mediated dephosphorylation of CSPD^{R} was recorded on Hyperfilm MP (Amersham, Arlington Heights, IL) and analyzed by densitometry (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, CA). To compare mRNA expression in different tissues, data were normalized to the β-actin expressed by that tissue.

The full cDNA of lipophilins A and B were used to screen a human placenta cosmid library (Clontech, Palo Alto, CA). Seven positive clones were chosen. Southern blots performed with specific oligonucleotide probes (P5, lipophilin A; P8, lipophilin B) identified 3 as lipophilin A clones and 4 as lipophilin B. A portion of the lipophilin C gene was obtained by amplifying human genomic DNA (Clontech) with P12 and P13. The cloned, ≈1.9 kb PCR product was found to encode the signal sequence and most of lipophilin C peptide, with an intervening intron. The entire genomic clones of lipophilin A, B and this partial lipophilin C gene were used to perform fluorescence *in situ* hybridization (see DNA Biotech, Toronto, Canada) on BrdU treated, phytohemagglutinin-stimulated normal human lymphocytes, as described by Heng and Tsui [11].

RESULTS AND DISCUSSION

Figure 1 shows the sequences of lipophilin A, lipophilin B, and rat prostatein C2. The 429 nucleotide (nt) lipophilin A sequence contained a 270 nt open reading frame encoding a 90 residue, 9,888.0 Da propeptide. Removal of its signal sequence would leave a 69 residue peptide whose predicted mass (7572.1 Da) equals

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PrC1  QICELVAHETISFLMKSEEELKKELEMYNAPPAAVEAKLEVKRCVDQMSNGDRLVVAETLVYIFLECGVKQWVE..
LpnA  VVCQALGSEITGFLLAGKPVFKFQLAKFKAPLEAVAAKMEVKKCVDTMAYEKRVLITKTLGKIAEKCDR
LpnB  EFCPALVSELLDFFFISEPLFKLSLAKFDAPPEAVAAKLGVKRCTDQMSLQKRSLIAEVLVKILKKCSV
PrC2  GVCQALQDVTITFLLNPEEELKRELEEFDAPPEAVEANLKVKRCINKIMYGDRLSMGTSLVFTMLKCDVKYGYK

PrC3  GSGCSILDEVIRGTINSTVTLHDYMKLVKPYVQDHFTEKAVKQFKQCFLDQTDKTLENVGVMMEAIFNSESCQQPS
LpnC  DSGCKLLEDMVEKTINSDISIPEYKELLQEFIDSDAAAEAMGKFKQCFLNQSHRTLKNFGLMMHTVYDSIWCNMKSN
Mmg   GSGCPLLENVISKTINPQVSKTEYKELLQEFIDDNATTNAIDELKECFLNQTDETLSNVEVFMQLIYDSSLCDLF
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FIG. 3. Primary amino acid sequences of lipophilins and some related peptides. The following sequences are shown: PrC1, PrC2 and PrC3 signify rat prostatein components 1, 2 and 3; Lpn A, LpnB and LpnC signify human lipophilins A, B and C; Mmg, human mammaglobin. Identical residues are connected by a vertical line, similar residues by a plus sign. Conserved cysteines are bolded.

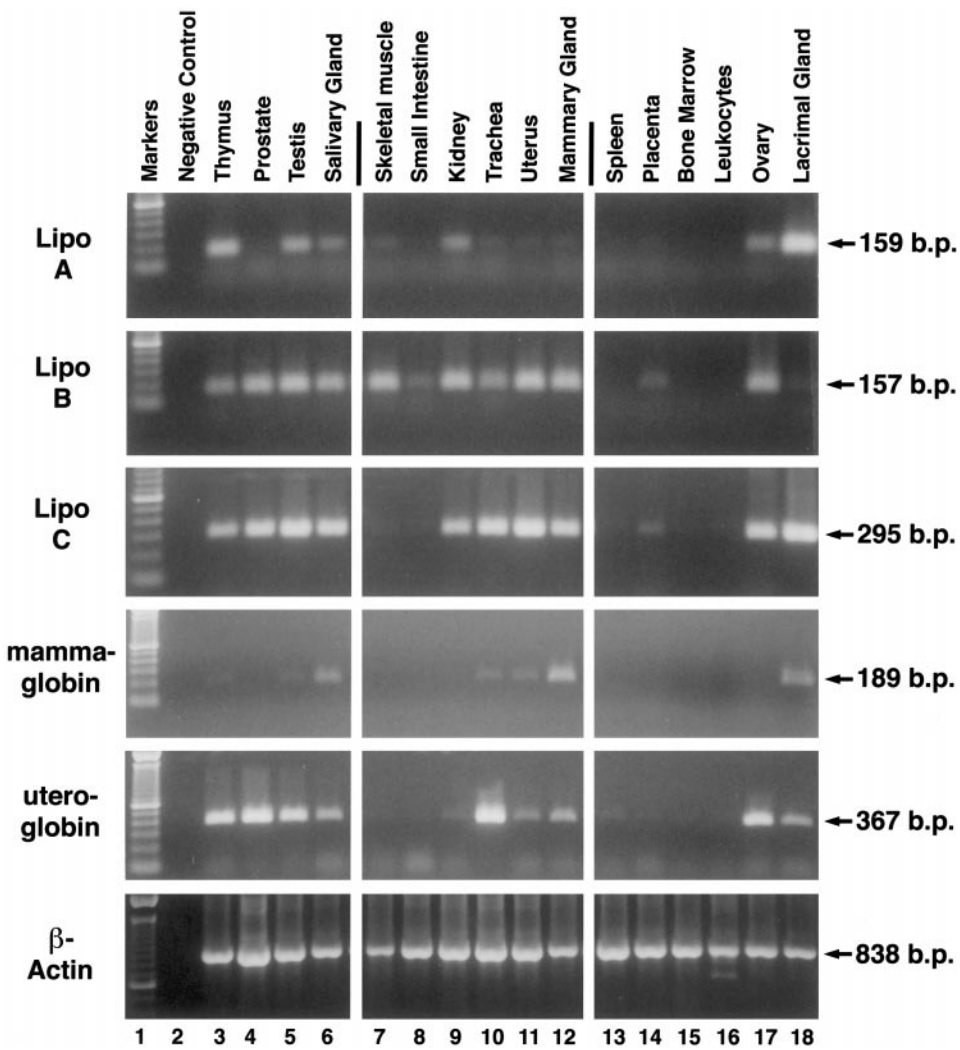

FIG. 4. Tissue expression. Expression of lipophilins A, B, and C, mammaglobin, uteroglobin and β-actin was examined by RT-PCR in 16 tissues that are identified in the figure. The primers, described in Table 1, were: lipophilin A, P5 + P6; lipophilin B, P8 + P9; Lipophilin C, P12 + P13; mammaglobin, P15 + P16; uteroglobin, P18 + P19.

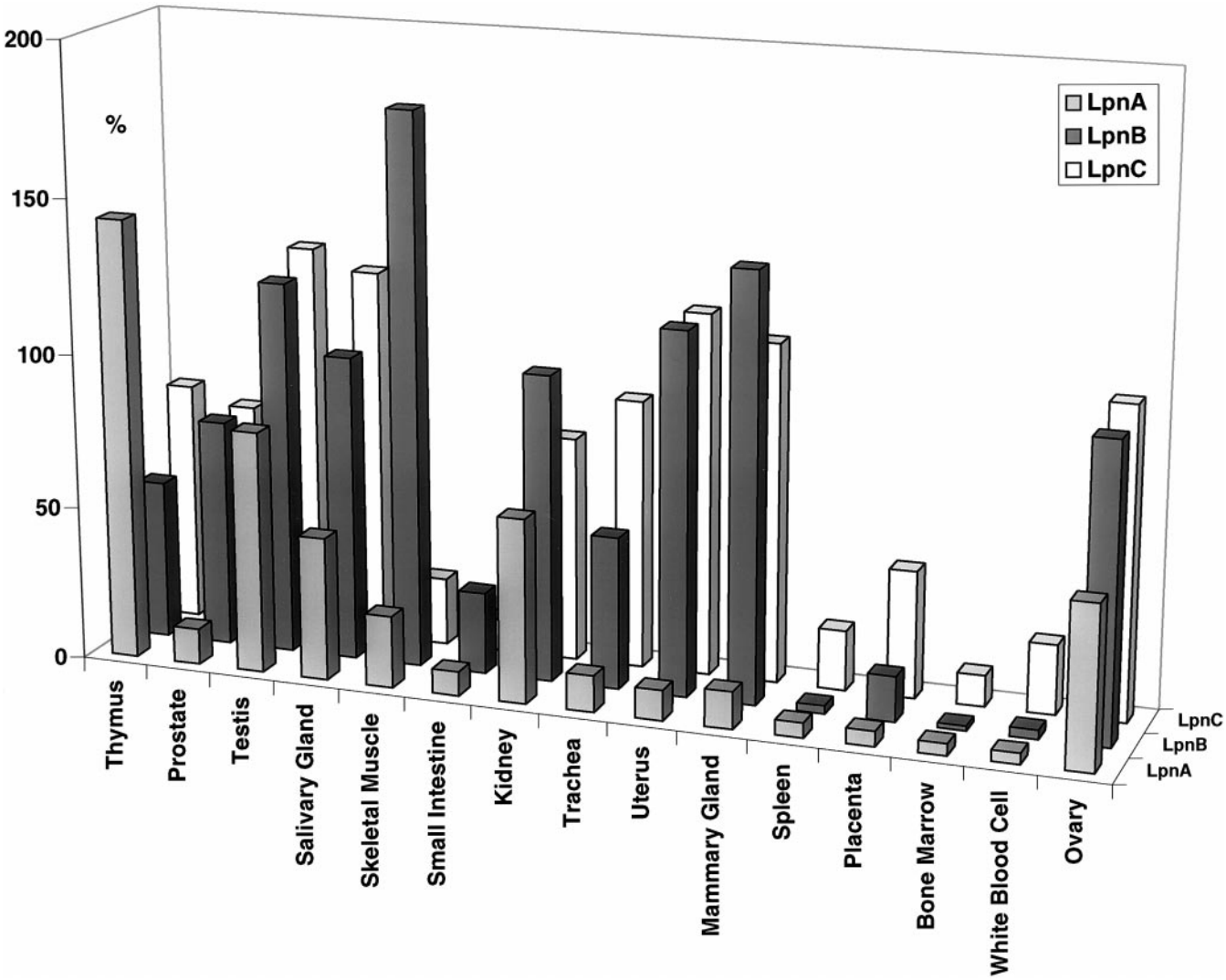

FIG. 5. Quantitative analysis of lipophilin expression. In this figure, expression has been normalized to that of β-actin in the same tissue.

that of the lipophilin A peptide (7,574.7 Da) that we purified from human tears [5]. Although lipophilin B has not yet been purified as a peptide, its cDNA sequence contains a putative initiating methionine codon at nt 83- 85 and a stop codon at nt 353-355. The 270-bp open reading frame encodes an 90 amino acid precursor with a 69 residue mature peptide whose predicted masses are 9,925 and 7769.2 Da, respectively.

The nucleotide sequences of lipophilin C, rat prostatein C3, and human mammaglobin [6] are shown in Fig. 2. The 285 nt open reading frame of lipophilin C starts at nt 41 and predicts a 95 residue propeptide (10,879.8 Da) with an 18 residue signal sequence. The predicted mass (8,851.1 Da) of the 77 residue mature peptide agrees with the lipophilin C peptide we purified from tears (8,854.94 Da). This sequence is 99.6% identical (492/494 bases) to that of mammaglobin B (**7,** EMBL GenBank AF0721219). Figure 3 shows the deduced amino acid sequences of lipophilins A, B and C, and their homology to mammaglobin, and the components of rat prostatein.

We used RT-PCR to examine expression of lipophilin components A, B and C in various tissues (Fig. 4). Mammaglobin, uteroglobin (also called CC-10) [8] and β-actin served as controls. Mammaglobin expression was especially prominent in breast, but also occurred in salivary and lachrymal gland tissue. Uteroglobin/CC-10 expression was prominent in thymus, trachea and steroid-responsive tissues (prostate, testis, uterus, breast, and ovary), and occurred to a lesser extent in lachrymal and salivary glands. Expression of lipophilin C (mammaglobin B) was widespread, occurring in all tissues that expressed uteroglobin, plus the kidney, which did not. Lipophilin C expression exceeded that of

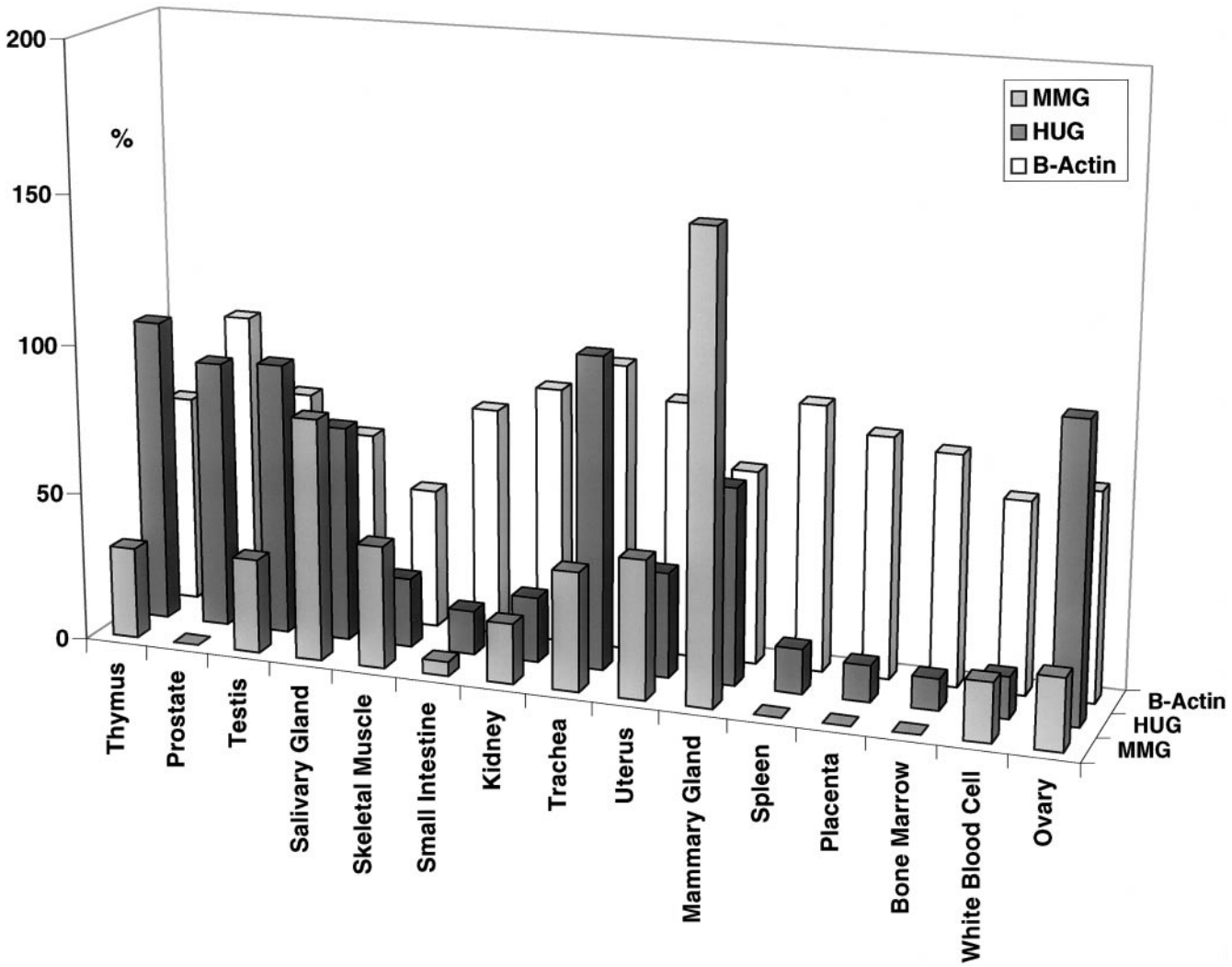

FIG. 6. Quantitative analysis of mammaglobin and uteroglobin expression. The expression of mammaglobin (MMG) and uteroglobin (HUG) is normalized to that of β-actin in the same tissue. β-actin expression is expressed relative to that of the highest sample value (prostate gland).

uteroglobin in the mammary, lachrymal and salivary glands and the uterus. Lipophilin B expression generally paralleled that of lipophilin C, but was less prominent in trachea and ovary. Lipophilin B was expressed in salivary but not in lachrymal gland. Expression of lipophilin A was most prominent in lachrymal gland, substantial in thymus, moderate in salivary gland and virtually absent in the other organs sampled.

Tissue expression was assessed quantitatively by normalizing each peptide's expression to that of β-actin in the same tissue (Fig. 5). The sex steroid-responsive organs (prostate, testis, uterus, mammary gland and ovary) showed substantial and approximately equal expression of lipophilin components B and C. Considerable lipophilin A expression was found in thymus, and low level expression was present in the testis, kidney and ovary. Lachrymal tissue was not represented in this panel. Skeletal muscle expressed high levels of lipophilin B, without commensurate expression of lipophilins A or C. None of the three lipophilins showed appreciable expression in the bone marrow, white blood cells, spleen or small intestine.

Uteroglobin expression was prominent in the prostate, testis, ovary, mammary gland, salivary gland, trachea and thymus (Fig. 6). As expected, mammaglobin mRNA was most abundant in the mammary gland. However, substantial expression occurred in salivary gland, and some expression was seen in other organs that also expressed uteroglobin. To confirm the extramammary expression of mammaglobin, we cloned and sequenced PCR products obtained from primary cultures of human prostatic and tracheal/bronchial epi-

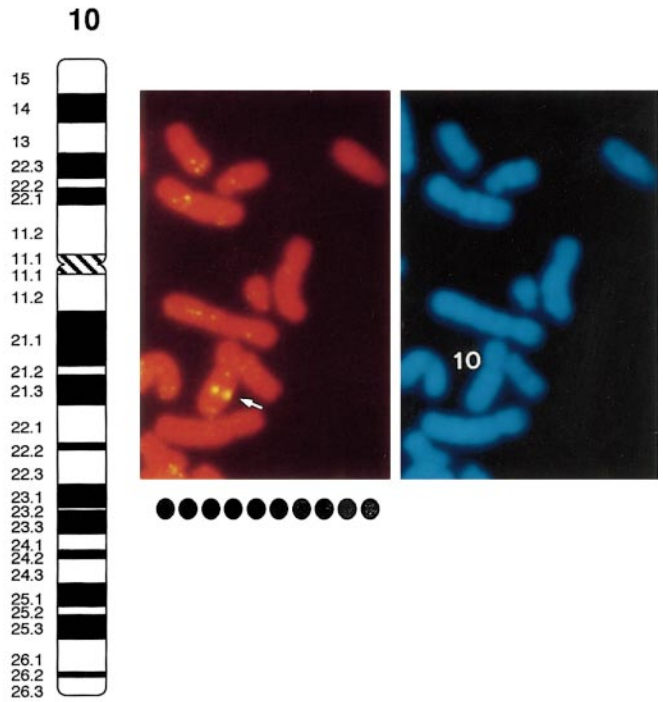

FIG. 7. Chromosomal location of the lipophilins. The genes were localized by fluorescent *in situ* hybridization (FISH) [11]. Only the study with lipophilin B is shown. Left: The yellow spots in the left color panel are paired genes on chromosome 10, a pattern seen in 94/100 mitoses. The right panel shows a DAPI stain of the same mitotic figure. The diagram summarizes detailed mapping studies of ten miotic figures, showing localization to 10q23.2.

thelial cells (Clonetics), and found them to be 100% identical to the described mammaglobin sequence (data not shown).

Human metaphase chromosomes were hybridized individually with biotinylated probes consisting of lipophilin A and B cosmids or the lipophilin C probe described above, and the complexes were detected with avidin-FITC. Of 100 mitotic figures, 93% (lipophilin A), 94% (lipophilin B), and 71% (lipophilin C) showed signals on the long arms of a single chromosome pair, identified by DAPI binding as chromosome 15 (lipophilin A), chromosome 10 (Lipophilin B), and chromosome 11 (Lipophilin C). The detailed positions were more accurately determined from 10 photomicrographs each to be: 15q12-q13 (lipophilin A), 10q23 (lipophilin B), and 11q12-q13.1 (lipophilin C). Becker et al also localized the lipophilin C (mammaglobin B) gene to chromosome 11 q13 (7). Only the study with lipophilin B is shown in Fig. 7.

A dendrogram, anchored by mouse lachrymal gland peptide (GenBank AF008595, Lima *et al.*), shows the relationships between these peptides and other members of the uteroglobin superfamily (Fig. 8). Lipophilins A and B are related most closely to each other, and next to the C1 and C2 components of rat prostatein. Lipophilin C (mammaglobin B) and mammaglobin are most closely related to each other, and next to the C3 component of prostatein. Human [8], rabbit [12] and mouse [13] uteroglobin molecules (HUG, RUG and MUG) are more distant relatives of the aforementioned peptides.

Like human mammaglobin and rat prostatein components C1, 2 and 3, lipophilins contain three invariantly conserved cysteine residues (Fig. 3). Rat prostatein molecules are tetramers containing one C1|C3 heterodimer and one C2|C3 heterodimer, in a noncovalent association. To date, mammaglobin has been studied only in terms of its mRNA expression and gene structure [6, 9]. It remains to be determined if human mammaglobin, like lipophilin C, binds covalently to either lipophilin A or B.

Whereas prostatein and lipophilins contain three conserved cysteines and form mixed dimers, uteroglobin monomers contain only two and form homodimers [14]. Because the C1 and C2 components of rat prostatein have different steroid binding properties [3], formation of mixed dimers could create molecular assemblages that bind different lipid ligands than those accommodated by homodimeric, uteroglobin-like molecules.

Localization of the lipophilin B gene to chromosome 10, band q23 is of considerable interest. Not only does chromosome 10q22-23 harbor an anti-oncogene strongly implicated in prostate cancer [15], this region has also been implicated in Cowden syndrome, an autosomal dominant hamartoma syndrome associated with a high risk of thyroid and breast cancer [16, 17]. Although interest has centered on PTEN/MMAC1 [10, 18, 19], evidence [20] suggests that additional prostatic cancer tumor suppressor genes may be situated close to the PTEN/MMAC1 locus on 10q23. It will be important to determine if lipophilin B plays such a role. Both the mammaglobin gene and the lipophilin C (mammaglo-

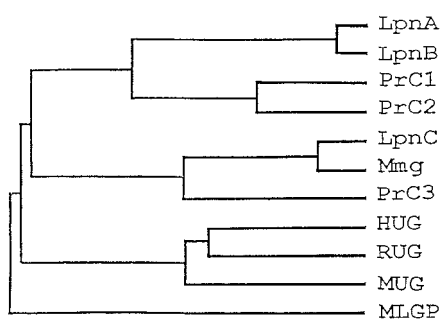

FIG. 8. Dendrogram. The figure, based on nucleotide sequences of the propeptides, was constructed according to the method of Myers and Miller [24]. Lipophilins A, B and C are described in this report. PrC1, PrC2 and Pr C3 signify rat prostatein components 1, 2 and 3. HuG, RUG ands MUG stand for human, rabbit and mouse uteroglobins [25]. MLGP signifies mouse lachrymal gland protein (GenBank AF008595) [26].

TABLE 1

Primer Sequences for cDNA Cloning, PCR Amplification and Southern Blot

cDNA	Primers	Sequences (5′-3′)		Location (bp)
LpnA	P1	AAAATGGAA/GGTIAAA/GAAA/GTGT/CGT	Sense	222–244
	P2	TCA/GCAT/CTTTT/CTCIGCAATTTT	Antisense	313–293
	P3	CCCAATGTTTTTGTAATTAGCACTC	Antisense	292–268
	P4	ATCACTCATCATTGGTTAAAGCCGAGCTC	Sense	1–29
	P5	GAAATCACAGGCTTCTTATTAGCTG	Sense	135–159
	P6	TCCCAATGTTTTTGTAATTAGCAC	Antisense	293–270
	P7	GTGGATACGATGGCCTATGAGAAAAG	Sense	243–268
LpnB	P8	GCTGTTAGACTTCTTCTICATTAGTG	Sense	172–197
	P9	CACCAGGACTTCCGCAATGAGGC	Antisense	328–306
	P10	ACGGATCAGATGTCCCTTCAGAAACG	Sense	278–303
LpnC	P11	TCT/GGAGTTIATT/GGTTTTT/CTC	Antisense	147–128
	P12	CTGCCACGCACGACTGAACACAGAC	Sense	1–25
	P13	GTACACTGTATGCATCATCAGTCCAAAG	Antisense	295–268
	P14	AAACTCCTGGAGGACATGGTTGAA	Sense	107–130
Mmg	P15	ATATATTAATTGCATAAACACCTCAACA	Antisense	315–288
	P16	CCCTTATTGGAGAATGTGATTTCC	Sense	127–150
	P17	CCACTACAAATGCCATAGATG	Sense	221–241
HUG	P18	CTCCACCATGAAACTCGCTGTCACCC	Sense	1–26
	P19	GAAGAGAGCAAGGCTGGTGGGCGTGG	Antisense	367–342
	P20	CAGCTGAAGAAGCTGGTGGACACCC	Sense	188–212

bin B) gene are localized to chromosome 11q13 [7], a region that is frequently amplified in breast neoplasms [9]. Their homology and shared location indicate that they arose relatively recently by gene reduplication.

Assuming that the human lipophilins are the functional counterparts of rat prostatein, their properties may include an ability to bind androgens and other steroids, and transcriptional regulation by steroid hormones [21]. They may also bind and concentrate estramustine [22, 23], a chemotherapeutic agent widely used for prostate cancer. Further studies are needed clarify the biological roles of these intriguing peptides. The information presented in this report should enable these hypotheses to be tested experimentally.

ACKNOWLEDGMENTS

The cDNA sequences of lipophilins were deposited with EMBL Nucleotide Sequence Database, with the following accession numbers: Lipophilin A, AJ224171; Lipophilin B, AJ224172; Lipophilin C, AJ224173.

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